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Macromolecular Synthesis of Cellulomonas Grown in Continuous Culture.

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MACROMOLECULAR SYNTHESIS OF *CELLULOMONAS* GROWN IN CONTINUOUS CULTURE

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ABSTRACT

Steady-state continuous culture was used to optimize a lean chemically defined medium for Cellulomonas LC-10. The organism was extremely sensitive to variations in trace metal concentrations. Medium optimization by this technique proved rapid and multi-factor screening was easily conducted using a minimum of instrumentation. The optimized medium supported a critical dilution rate of 0.571 h^{-1} which approximated the maximum specific growth rate observed in batch culture.

The organism was cultivated under glucose and zinc limitation at a variety of growth rates in continuous culture. The growth characteristics and macromolecular composition of the population varied with the limitation imposed and growth rate. Glucose and zinc-limited cultures maintained a constant relative protein content. The relative RNA content increased, whereas the carbohydrate and DNA contents decreased with an increase in the population growth rate in glucose-limited cultures. Free unbound lipid remained constant. The maximum population growth rate in zinc-limited cultures was directly proportional to the zinc concentration and

demonstrated a traditional steady state function. The nucleic acid content increased with increased growth rate, however relative nucleic acid content was significantly depressed when compared to glucose-limited cells. This manner of cultivation may prove to be a useful tool for the production of single cell protein with lowered nucleic acid content and the elucidation of micronutrient involvement in growth related processes.

In addition, a gradient-feed technique was developed and successfully tested to achieve rapidly a high cell density culture at the bench scale level. Cell yields of approximately 30 g/l dry weight were achieved in 10 h.

INTRODUCTION

The salient feature of members of the genus Cellulomonas is the ability to attack cellulose. For this reason members of this laboratory have been examining wild type and mutant strains of a Cellulomonas sp. as a potential source of single-cell protein (Han, 1969; Fleenor, 1973; Choi, 1976). The primary goal of our studies is to provide basic research information to aid in the design and optimization of such a fermentation process. A process for the conversion of cellulose to a nutritional product is economically feasible since the substrate is inexpensive and available. Information regarding the growth characteristics and macromolecular composition of Cellulomonas must be gained in order to estimate the maximum potential available for development. Continuous cultivation can provide a means for examining these features as a function of population growth rate.

The development of an industrial process presents a variety of unique problems. Since the primary product is biomass, the environmental conditions must be optimized to maintain a rapid population growth rate. Associated with rapidly growing cultures is an elevated

nucleic acid content, and a reduction of nucleic acids in both wide whole cells and isolated protein is desired (Chen and Peppler, 1978). Currently, this can be accomplished by reducing the population growth rate (Chen and Peppler, 1978), activating endogenous nucleases (Canepa, et al., 1972; Sinskey and Tannenbaum, 1975), and a variety of chemical extraction techniques (Decker and Dirr, 1944; Lindblom and Morgan, 1974; Vananuvat and Kinsella, 1975). Manipulations such as these, however, are often time consuming and expensive. Since the presence of high levels of nucleic acids is intrinsically related to growth, an understanding of the interaction between the growing population and the environment may provide information enabling the reduction of nucleic acid content without severely reducing productivity.

This dissertation describes the optimization of a lean chemically defined medium for the growth of Cellulomonas LC-10 in continuous culture. The growth characteristics and macromolecular composition was determined in continuous culture under glucose and zinc limitation at a variety of growth rates. In addition, a method for the achievement of high cell density cultures is described where the microbial population and

nutrient concentration of the medium is kept in balance to extend the phase of exponential growth.

REVIEW OF LITERATURE

I. Perspectives of bacterial growth.

A. Batch culture growth cycle.

The cornerstone supporting the foundation of microbiological research is the understanding of the bacterial batch culture growth cycle. Throughout the course of scientific history researchers have attempted to describe the entire cycle both mathematically and physiologically. The early descriptions have been accepted as traditional standards depicting the "normal" bacterial culture characteristics. However, subtle variations in environmental or nutritional conditions have produced alterations in population growth responses which are still unclear. To date, complete scientific understanding has not been achieved.

The events of batch culture growth were described as mathematical functions by Buchanan (1918) who examined the results of previous workers. His treatment extended the recognition of 4 universally accepted phases to 7 distinct phases designated as follows:

1. Initial stationary phase - Following inoculation of cells into a suitable medium there is an interval of time before a cell will resume growth sufficiently to divide. Therefore the number of bacteria will remain constant during this period and, when plotted as a function of time, lie in parallel to the X axis. During this time the average generation time is infinity.

2. Lag phase, or positive growth acceleration phase - This phase is usually not separated from the previous phase by most investigators. The need to differentiate the two phases may not be required, but it clearly does reflect two different mathematical events of the growth cycle. During this phase the average rate of increase in numbers per organism increases with time to a certain point designated the minimum generation time. The general equation derived to describe lag growth is

$$b = Be^{kt^n}$$

where

b = number of bacteria after time t,

B = initial number of bacteria,

k = velocity coefficient of growth,

n = number of generations in time t.

In actual practice, there are two principle ways in which a mathematical expression can be used to indicate the amount of lag. First, an expression may be used which directly describes the length of lag period. This is termed the "period of lag measurement" and is routinely used. Second, an expression may be presented which assigns a numerical value to the degree of depression of rate of multiplication at any time during the progress of the lag period, and is termed the "time index of lag." Buchanan (1918) continued to explain the two principle ways in which a numerical value can be assigned to the time index of lag; by determining the ratio of the generation time at any instant to the minimum generation time of the logarithmic phase, or, the rate of increase per organism at any given instant during lag compared to the similar rate of increase during the logarithmic period.

When the physiological characteristics of growth are examined, the lag period is usually defined as that period of time from the moment of inoculation to the establishment of a constant and maximum rate of cell division (Winslow and Walker, 1939; Herbert, 1961; Stephenson, 1974). Early work reviewed by Winslow and Walker (1939) supported the contention of a biological basis for the phenomenon of lag on the basis of the

following four observations: cultures of differing age transferred to fresh medium demonstrated differing generation times, selected parameters of chemical activity were greater during early phases of the culture cycle than at later phases, culture resistance was greater during early phases compared to the later phases, and cellular morphology often changed during the culture cycle. Herbert (1961) pointed out that when cells are inoculated into fresh medium, exponential growth (as measured by increase in dry weight) begins almost immediately, but there is no corresponding increase in cell numbers for a considerable period. During this period of "division lag" there is a rapid increase in cell size and even more a rapid increase in RNA content. Stephenson (1974) contended that the increase in cell material was due to something in fresh media which was inhibitory to cell division. The contention is supported by observations that large inocula or diluted concentrations of media tended to decrease the cell size effect and lag time. Recently, it has been shown that inoculum size affected the length of lag time in two ways. In one case the lag time was reduced regularly, and in the other case the reduction was irregular. In addition, the medium composition and

supplementation of nutrients influenced the reduction of lag time, but the effect of the compounds used varied from strain to strain (Shida, et al., 1975). Properties associated with the lag phase are poorly understood and that the expression of growth rate based upon increasing bacterial number is probably inaccurate.

3. Logarithmic growth phase - During this phase the average growth rate per organism remains constant and is termed the minimal generation time (Buchanan, 1918). This phase has been investigated in depth compared to the other phases because any change in environmental conditions acts upon the rate of bacterial increase and will be manifested through a change in the generation time (g). The mathematical relationship presented by Buchanan (1918) to describe the log phase is

$$b = Be^{kt}$$

where k is related to the minimum generation time as follows:

$$k = \frac{\ln 2}{g}$$

and the equation for the growth curve is

$$b = Be^{kt} = Be^{(t \ln 2)/g}$$

Herbert (1961) points out that during logarithmic growth the cellular dry weight and numbers increase at the same rate while cell size, RNA per unit weight, DNA per unit weight, and protein per unit weight all remain constant. The assumption that is made is that all cells are dividing and viable, and division is occurring at a constant rate. Thus, kinetic investigations on cultures of microorganisms are suited for establishing relations between growth and environmental factors, especially the nature and amount of nutrients (Van Neil, 1974). Contrary to the accepted mathematical description, Stephenson (1974) pointed out that as early as 1922 discrepancies were observed between total particle counts and total viable counts in actively dividing cultures. The results supported a contention that during every generation the majority of organisms divide while a constant percentage (approximately 20%) fail to do so. If 20% of the population failed to divide each generation the logarithmic plot of viable cells would run parallel to a logarithmic plot of the total count and the actual mathematical description to solve for the number of generations would be changed from

$$n = \frac{\log b - \log B}{\log 2}$$

to

$$n = \frac{\log b - \log B}{\log 1.6}.$$

4. Phase of negative growth acceleration - Buchanan (1918) recognized this period as a result of a decrease in the growth rate per organism or an increase in the average generation time. Bacteria do not maintain their maximum growth rate for long periods of time, usually as a result of toxic product accumulation, nutrient limitation, cellular differentiation to a resting state, and cellular death. The relationship is complex, but the equation for growth can assume the form

$$b = Be^{kt-n}.$$

5. Maximum stationary phase - This phase is depicted as practically no increase in cell number and the change in cell number versus time plots parallel to the X axis. It is mathematically equivalent to the initial stationary phase in which b = B (Buchanan, 1918).

6. Phase of accelerated death - This phase marks the decrease in bacterial numbers, beginning slowly at first and increasing until a maximum logarithmic rate is reached. Buchanan (1918) describes this mathematical expression as

$$b = Be^{kt-n}.$$

7. Logarithmic death phase - During this phase the rate of death per organism remains constant. If the logarithm of the number of surviving bacteria at various time intervals is plotted against time, the points will be found to lie on a straight line. Buchanan (1918) describes this as

$$b = Be^{-kt}.$$

The basic mathematical treatments proposed by Buchanan (1918) have been accepted and withstood the tests of time. At best the formulae serve to describe the events of the batch culture, not explain them. Batch culture growth is a dynamic series of events continuously being altered by a changing environment. Since the environment is continuously changing the physiological response of the organism is also changing. It is for this reason that the use of continuous cultivation has been an important tool to aid investigators to gain an understanding of growth related physiological functions.

B. Theory of continuous cultivation.

The theory of continuous cultivation has been thoroughly reviewed (Monod, 1950; Herbert, et al., 1956; Tempest, 1970). Briefly, all continuous flow systems

consist of some form of reactor into which reactants flow at a steady rate and from which products emerge. Sterile growth medium is usually fed into the culture vessel at a steady flow-rate (f) and the culture emerges from the vessel at the same rate. The volume of the culture vessel (v) is held constant and the contents are well mixed so that the entire contents are equally distributed. Residence times in such a vessel are determined by the ratio of the flow rate and culture volume which is called the dilution rate, D, defined as D = f/v (the number of complete volume changes/hr.). The mean residence time of a particle in the culture vessel is equal to 1/D.

The most common theoretical expression for exponential growth is the equation

$$\frac{1}{x}\frac{dx}{dt} = \frac{d(\ln x)}{dt} = \mu = \frac{\ln 2}{t_d}$$

where x is the concentration of organisms (dry weight of organisms/unit volume) at time t, μ is the specific growth rate, and t_d is the doubling time. The terms u and t_d are usually throught of as constants, however, this is only a correct assumption when all the nutrients required for growth are present in excess (Herbert, et al., 1956). If the concentration of one nutrient is decreased to a low level, the specific growth rate is lowered correspondingly. The dependence of u upon the

substrate concentration (S) was shown by Monod (1942) to be a form represented by a Michaelis-Menten function

$$u = u_{max} \left(\frac{S}{K_s+S}\right)$$

where u_{max} is the maximum value of μ (when the substrate is not growth limiting) and K_s is the substrate saturation constant (substrate concentration equal to the growth limiting concentration at 0.5 μ_{max}). In batch culture all nutrients are present initially in concentrations not limiting the growth rate, thus exponential growth is generally equal to μ_{max} (Tempest, 1970). It follows that exponential growth can occur at a rate between zero and μ_{max}, as long as the substrate concentration can be held constant at an appropriate value. This is an important factor for the theory of continuous cultivation (Monod, 1950; Novick and Szilard, 1950a; Novick and Szilard, 1950b).

Monod (1942) also observed a constant relationship between rates of growth and substrate concentration, where

$$\frac{dx}{dt} = -Y\frac{ds}{dt},$$

and

$$Y = \text{yield factor} = \frac{\text{weight of bacteria formed}}{\text{weight of substrate consumed}}.$$

Thus, if the values of the three growth constants μ_{max}, K_s, and Y are known, a complete quantitative description of the growth cycle of a batch culture can be achieved (Monod, 1949).

When bacteria are growing in continuous culture all nutrients are in excess except one which is at a growth limiting concentration. All other environmental conditions are carefully controlled. During cultivation the net rate of increase of organism concentration is given by a simple equation:

$$\text{increase} = \text{growth} - \text{output},$$

or

$$\frac{dx}{dt} = \mu x - Dx.$$

If μ D, dx/dt is positive and the concentration of organisms will increase, while if μ . D, dx/dt is negative and the concentration of organisms will decrease, eventually to zero. When μ = D, dx/dt equals 0 and x is constant resulting in what is termed a steady state culture (Herbert, et al., 1956). Therefore, to accomplish continuous cultivation conditions must be provided where the specific growth rate (μ) and D are equal and invariable with time in which

$$D = \mu = \mu_{max} \left(\frac{S}{K_s+S}\right) = \frac{\ln 2}{t_d}.$$

This is not difficult to achieve because the population growth rate is limited by the rate of supply of the growth limiting nutrient and must be proportional to D as long as D is constant. Since μ is governed by D, μ can be adjusted within limits to any value, but cannot exceed μ_{max}. Therefore, steady state cultures cannot be maintained at dilution rates above a critical value (D_c) which is nearly equal to μ_{max} (Tempest, 1970).

Continuous cultivation, therefore, has become a powerful tool for examining growth associated physiological events by extending the range of culture conditions. By maintaining a stringent control over enviromental influences five basic advantages are associated with chemostat investigations (Pirt, 1973).

1. It provides a means of controlling growth rate.
2. The growth rate can be held constant while physical and nutritional conditions are changed.
3. It provides a means of achieving substrate-limited growth with a constant concentration of the limiting substrate.
4. The chemostat permits the biomass in a culture to adjust itself to a steady state in any given environment.

5. The chemostat permits the most rapid conversion of substrate into biomass.

II. Bacterial single-cell protein.

The concept of using microorganisms for food or feed uses is not novel. Algae, bacteria, yeasts, and fungi are consumed daily as yeast-leavened baked products, cheeses and fermented milks, fermented sausage products, mushrooms, and oriental fermented food products (Litchfield, 1978). The importance for innovative technological development towards the production of microbial proteins was not realized until the 1960's when a neck and neck race began between food production and population. Rising affluence has placed a major strain on world food sources due particularly to increased *per caput* beef consumption, overfishing and pollution of world fisheries, and decreased *per caput* legume production (Scrimshaw, 1975). Experimentation began for the purpose of developing an industrial fermentation for the production of single-cell protein (SCP) from a wide variety of procaryotic and eucaryotic cells cultivated upon inexpensive substrates. The majority of this work has been done using yeast and filamentous fungi, however, some bacterial systems show

promise. The major limitations about the use of SCP for direct human consumption are economic feasibility, safety, and palatability (Scrimshaw, 1975; Rha, 1975), but these limitations are relaxed when SCP is used as animal feeds (Shacklady, 1975; Duthie, 1975).

The interesting features about the use of bacteria as a potential SCP source compared to other sources is that they have a higher protein content and that their proteins show a well balanced amino acid composition (Rey, 1971). In addition, exponential bacterial growth rates are generally rapid which is a feature conducive to a system for the economical production of biomass. However, a high nucleic acid content is a characteristic of rapidly growing cells (Kihlberg, 1972) and is a major disadvantage.

Bacterial SCP production from methane has been examined using mixed (Harrison, 1976) and pure cultures (Hamer, et al., 1975; Harrison, 1976). A methane oxidizing Pseudomonas sp. was highly sensitive to methanol concentration in the medium and maximum yields could only be obtained in methanol-limited continuous culture. Yields could be increased to about 0.85g cells (dry weight) per g methane by including a methanol-utilizing Hyphomicrobium sp. and two further species capable of utilizing complex organic molecules and operating under both

oxygen and methane-limitation. It was evident that methanol was produced as an extracellular product by the methane-utilizing Pseudomonas. Methanol concentrations greater than 0.005 g/l inhibited methane oxidation. At rapid growth rates (0.55 h^{-1}) cell yields of 35 g/l were obtained at substrate conversions of approximately 0.47 g/l. The product contained 80% crude protein which was slightly deficient in the amino acid methionine (Harrison, 1976). These yields were significantly higher than those obtained from continuous mixed cultures isolated from sludge (Sheehan and Johnson, 1971). Yields of approximately 62% were observed over a range of dilution rates from 0.085 to 0.301 h^{-1}, however, the culture density was very low. Hamer, et al. (1975) have reviewed the studies conducted with Methylococcus capsulatus showing the achievement of high yields, but it was pointed out that high productivities were difficult to maintain.

The production of SCP from methanol has been examined by Cooney, et al. (1975) using continuous cultivaiton of a yeast. The authors recognized, however, that equally high productivity could be achieved using a Pseudomonas sp. The ICI process for the production of Pseudomonas sp. for SCP is run continuously at 40°C and

demonstrates yields of 0.5 g cells (dry weight) per gram of methanol. The cells contain approximately 83% crude protein (Gow, et al., 1975). The results achieved using Methylomonas methanolica are also promising, however, this has been examined using batch growth conditions only (Dostalek and Molin, 1975).

Fornery and Reddy (1977) reported the examination of a process for the production of a crude protein feed supplement by fermentative conversion of potatoe-processing wastes using lactobacilli. The process is interesting in that the substrate consists of starch waste and would be inexpensive, but the growth rates and yields were rather low. In addition, the waste water had to be supplemented with trace minerals, trypticase, and yeast extract to support growth of the organisms and the final product only contained 43.4% crude protein.

Cellulosic substrates are extremely attractive for the production of SCP because there are vast amounts available and it is continuously replenished (Dunlap, 1975). A variety of cellulolytic bacteria, such as Pseudomonas sp. (Thayer, 1976; Ramasamy, et al., 1979), Cellvibrio sp. (Berg, 1975; Breuil and Kushner, 1976), thermophilic Actinomyces (Bellamy, 1977), Cytophaga sp. (Chang and Thayer, 1975), and a Cellulomonas sp.

(Han, 1969), have been examined as a possible industrial source for SCP. Equally interesting has been the number of mixed culture systems that have been examined (Han, 1969; Srinivasan and Fleenor, 1972; Paredes-Lopez and Gonzalez, 1973; Paredes-Lopez, et al., 1974; Thayer, et al., 1975; Kristensen, 1978; Thayer, et al., 1978). Each of these studies demonstrated potentiality for development, however, rapid growth rates seen using soluble substrates were not achieved. Usually the organisms grow in batch culture very slowly. Also, the cost of pretreating cellulose to make it susceptible to enzyme hydrolysis is an important barrier toward further development (Dunlap, 1975).

III. Trace metal nutrition in microorganisms.

The importance of trace metals in the cultivation of microorganisms is often underestimated. Their presence in complex bacteriological media at acceptable levels is often taken for granted (Winder and Denneny, 1956). The list of ions which are considered to be essential at low levels includes Mg^{+2}, Ca^{+2}, Fe^{+2}, Zn^{+2}, Mn^{+2}, Co^{+2}, Cu^{+2} and Mo^{+2}. Concentrations of these micronutrients range from mg/L levels in the case of Mg^{+2} and Fe^{+2} down to ug/L levels as seen with Cu^{+2} and

Mn^{+2} (Silver and Krolovic, 1969). Walker (1954) showed that the oxidation state as well as concentration of a given ion was also important.

There are few studies reported in the literature which deal with optimum levels of micronutrients for growth of bacteria. Wilson and Reisenauer (1970) suggested that the scarcity of literature on the subject might be due to the inability to show an effect at low levels especially under conditions of batch cultivation. The majority of studies which deal with trace metal requirements of microorganisms have been focused on fungi, probably because of their economic importance in the fermentation industry. These studies have demonstrated that the manner in which substrate carbon is metabolized for production of primary metabolites is dependent upon the trace metal content of the medium. A striking example of such an effect is the involvement of zinc in citric acid (Sanchez-Marroquin, et al., 1970; Wold and Suzuki, 1976) and cynodontin (White and Johnson, 1971) production. In both cases the cultures must be deficient in zinc for product formation. Optimum zinc concentrations for growth resulted in a significant reduction of the desired product. Conversely, prophyrin synthesis and 8-amino-levulinate dehydratase activity have been found to be

directly proportional to zinc concentration (Komai and Neilands, 1968).

The effect of trace metal concentration upon bacterial growth was demonstrated by Wilson and Reisenauer (1970). Five strains of a Rhizobium sp. were found to have growth maxima at concentrations of zinc and manganese which varied 1000 fold for each. It is important to have a sensitive experimental system to examine subtle physiological alterations due to environmental change within a narrow range of nutrient concentrations. The use of a chemostat is particularly advantageous since the population density is dependent upon nutrient concentration and the population growth rate is dependent upon the rate of supply of some limiting nutrient. Changes in micronutrient concentration may affect the population growth characteristics and result in an altered steady state population.

MATERIALS AND METHODS

I. Microorganisms and Methods.

All experiments were conducted with a Cellulomonas sp. (ATCC 21399) and a spontaneous mutant designated LC-10. Stock cultures were maintained at ambient temperature on nutrient agar slopes supplemented with 0.1% (w/v) yeast extract. The organisms were transferred monthly. Approximately every three months each organism was passed through and reisolated from a batch medium containing cellulose to ensure cellulolytic activity.

II. Media.

The parent Cellulomonas strain required the presence of yeast extract for growth. The Cellulomonas mutant required biotin and thiamine for growth. Pantothenic acid was not required, but its presence in the medium stimulated growth. Three defined media developed for the batch cultivation of LC-10 are described in Table 1. The ingredients comprising B-medium were selected for use in optimization studies in continuous culture.

Table 1. Chemical composition of three defined media for the batch growth of Cellulomonas LC-10. The initial pH of each medium was 7.2.

Ingredients	Concentration (g/liter)		
	CH-Medium	A-Medium	B-Medium
Glucose	5	5	5
$(NH_4)_2SO_4$	0.5	3	3
NaCl	-	1	-
K_2HPO	1.5	2	1.5
$NaH_2PO_4 \cdot H_2O$	0.5	-	0.5
$CaCl_2 \cdot 2H_2O$	0.05	0.01	0.1
H_3BO_3	-	$1x10^{-4}$	$1x10^{-4}$
$MgCl_2 \cdot 6H_2O$	0.1	0.5	0.5
$FeSO_4 \cdot 7H_2O$	$5x10^{-3}$	$5x10^{-3}$	$5x10^{-3}$
$ZnSO_4 \cdot 7H_2O$	$1x10^{-4}$	$1x10^{-4}$	$1x10^{-4}$
$COCl_2 \cdot 6H_2O$	$5x10^{-5}$	$5x10^{-5}$	$5x10^{-5}$
$CuSO_4 \cdot 5H_2O$	$5x10^{-6}$	$5x10^{-6}$	$5x10^{-6}$
$MnCl_2 \cdot 4H_2O$	$5x10^{-6}$	$5x10^{-6}$	$5x10^{-6}$
Sodium Citrate	0.3	-	-
Thiamine HCl	$10x10^{-6}$	$5x10^{-2}$	$5x10^{-3}$
D-Pantothenic Acid	-	$5x10^{-2}$	$5x10^{-3}$
D-Biotin	$5x10^{-6}$	0.2	$2x10^{-4}$
Generation Time (h)	3.03	2.10	1.82[a]

[a]Additional experiments using B-Medium indicate a minimum generation time of 1.50 - 1.55 h.

III. Measurement of population density.

The population density was suitably diluted in 0.1M phosphate buffer (pH 6.8) and turbidity measured using a Klett-Summerson photoelectric colorimeter, model 800-S, with a No. 66 red filter. Viable counts were determined by plating samples on nutrient agar supplemented with 0.5% (w/v) yeast extract following dilution in sterile 0.1M phosphate buffer (pH 6.8). Biomass was determined gravimetrically be washing cell pellets three times with distilled water and drying at 80°C to a constant weight in preweighed aluminum pans.

IV. Respirometry studies.

Early attempts towards medium optimization were conducted under batch growth conditions using a Gilson Differential Respirometer to measure differences in oxygen uptake due to changes in medium composition. Batch cultures of Cellulomonas were grown at 30°C, agitated at 200 RPM, in a medium consisting of basal salts solution, 0.1% (w/v) yeast extract, and 0.5% (w/v) carbon source. Stationary phase cells were harvested by centrifugation at 12,000 x g for 30 min. at 1-4°C. The cell pellets were washed once with sterile salts

solution, collected by centrifugation, pooled, and adjusted to the proper cell density by dilution with sterile salts solution. Two ml of cells were incubated in sterile medium lacking the additive component and carbon source until the temperature equilibrated. Following the addition of the remaining medium component the rate of oxygen consumption was measured directly by the change in volume caused by the uptake of oxygen and the absorption of released carbon dioxide into 0.2 ml of 20% (w/v) KOH located in the center well of each assembly.

V. Ribonucleic acid synthesis.

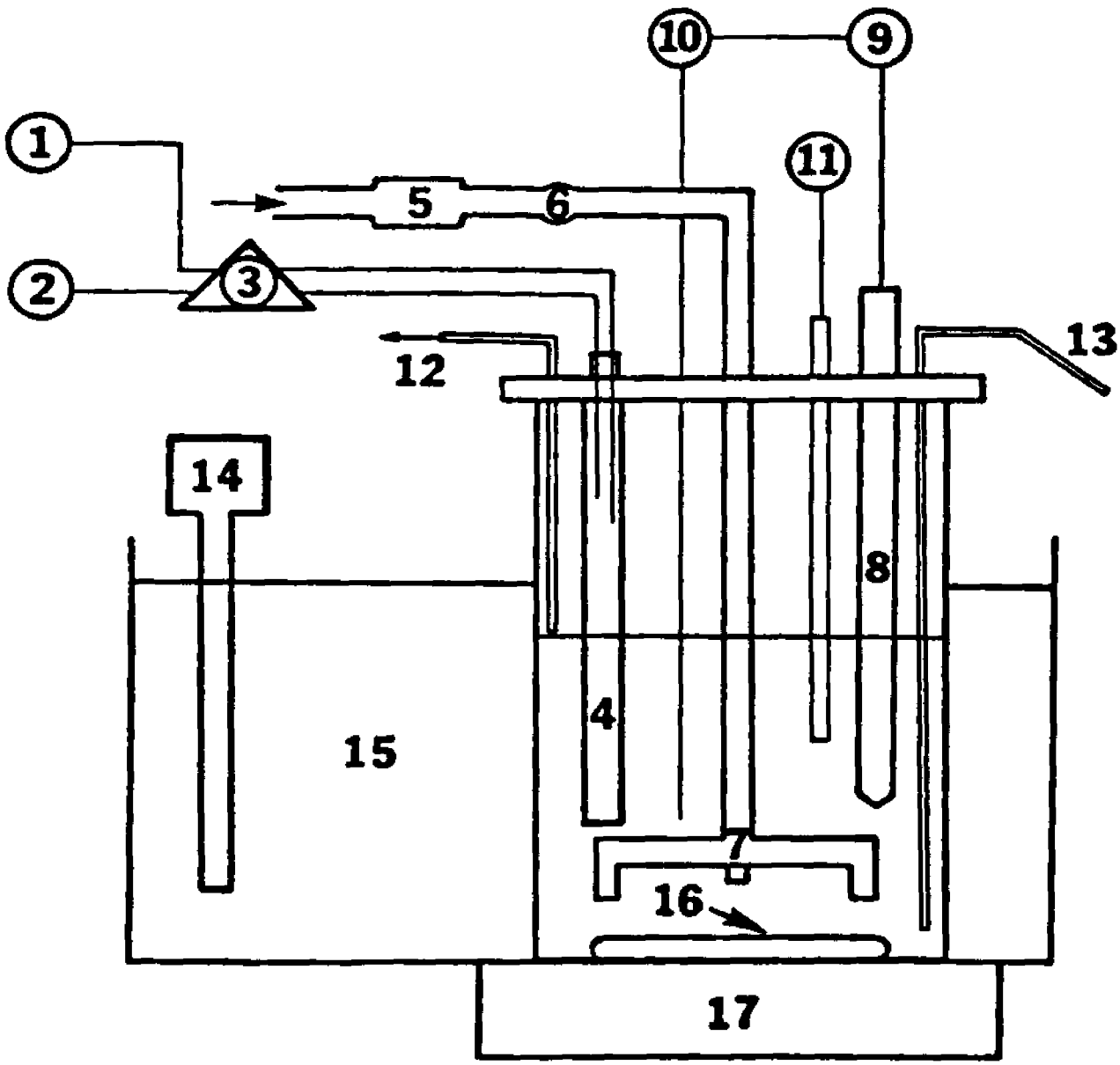
The rate of ribonucleic acid synthesis was measured by incorporation of [^{3}-H]-uridine into trichloroacetic acid (TCA) insoluble material. Cells were harvested from an overnight starter culture, washed, and resuspended in fresh medium to a culture density of 60 Klett units. The culture was incubated as previously described and at various intervals during the bacterial growth cycle 1 ml of culture was removed and placed into 100 ul of fresh medium containing [^{3}H]-Uridine (final concentration in the culture, 0.025 Ci/ml). The mixture was incubated under similar conditions and 100 ul samples were taken at 0, 1, 2, 5, and 10 min. intervals. Each

sample was mixed with 1 ml 10% (w/v) cold TCA and allowed to stand for 20 min. in the cold to allow for any equilibration of a uridine pool. The mixture was filtered through TCA presoaked filters (0.45 u, Selectron) and washed with 10 ml of TCA. Each filter was placed in a scintillation vial, dried at 30°C for 2 h, and mixed with 10 ml of scintillation fluid. The scintillation fluid consisted of the following: dioxane, 1000 ml; absolute methanol, 100 ml; napthalene, 60 g; 2,5-diphenyloxazole, 4g; 1, 4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 0.2 g. Each sample was counted twice for 20 min. in a Beckman Liquid Scintillation Counter.

VI. Continuous culture.

Influences of nutrient components were determined by use of a chemostat with a culture volume of 500 ml, as shown in Fig. 1. The fermentor consisted of a 1-liter Berzelius beaker sealed at the top with a silicon rubber stopper. Agitation was provided by a 58 mm x 19 mm star magnetic stirring bar and a magnetic stirrer. The fermentor was surrounded by a water bath and the temperature was controlled at 30°C ($\pm$ 0.2°C) using a Thermomix Circulator, model 1420 (V.W.R. Scientific, Inc.). For

Figure 1. Schematic diagram of chemostat. 1, Glucose reservoir constituents; 2, trace metal reservoir constituents; 3, peristaltic pump; 4, medium input tube; 5, coarse air filter; 6, microbiological air filter; 7, ground glass air spargers; 8, pH probe; 9, pH controller; 10, 0.5M NaOH and pump; 11, dissolved oxygen probe and recorder; 12, volume control aspirator; 13, sampling port; 14, thermoregulator; 15, constant temperature water bath; 16, magnetic stirring bar; 17, magnetic stirrer.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aeration, filter sterilized compressed air was introduced through 3 coarse ground glass spargers located near the bottom of the fermentor vessel. The dissolved oxygen concentration was monitored with an oxygen electrode (New Brunswick, M-1016-208) and Heath Servo-Recorder (model EU-20B), and did not fall during the experiments below 50% that attainable in fully aerated uninoculated medium. When required, the dissolved oxygen concentration was increased by increasing the aeration rate. The medium components were divided between two reservoirs to aid in identifying limiting nutrients. The medium in one reservoir contained glucose, vitamins, phosphate and nitrogen sources, whereas that in the other contained all the trace metals. The medium was acidified to prevent precipitation and 0.2 ml Polyglycol P-2000 was added to the glucose reservoir to prevent foaming. The components in both reservoirs were pumped at the same rate using a peristaltic pump (Harvard Apparatus Co., model 551) and were introduced near the bottom of the reactor vessel to insure adequate mixing. This was accomplished by shielding the two medium input lines with a glass tube located near one of the air spargers. Thus, the tube was kept filled with air and the medium dripped out the tube near the bottom of the fermentor. The theoretical flow rate was routinely checked by collecting

the culture effluent over a specific period of time and measuring its volume. No significant difference was noted at any time between the theoretical and actual flow rate. The pH of the culture medium was held between 6.8 to 7.2 using a Horizon pH Controller, model 5997 (Cole-Parmer, Inc.), and 0.5 M sterile NaOH. The culture volume was held constant by an aspirator.

VII. Medium optimization.

Medium optimization was carried out using Cellulomonas, LC-10. Cells used for inocula were washed from slopes and passed twice through 100 ml of B-medium in a 250 ml flask at 30°C and 200 RPM before introduction into the sterilized continuous culture medium. The cells were allowed to reach a glucose-limited steady state of growth at a slow dilution rate. The dilution rate was then increased to a point where glucose was no longer limiting. When this occurred the steady state population was considerably less than that obtained at the slower dilution rate. Glucose was detected in the effluent, and it was assumed that one or more of the other nutrients were limiting. The reservoir containing the limiting nutrient was identified by slightly increasing the concentration of all nutrients in first one carboy

and then the other, while keeping the glucose concentration constant. Once the proper reservoir was located, the original concentration was restored and the concentration of each nutrient was increased separately until the limitation was overcome and a glucose-limited steady state was achieved. The nutrient package thus established was substituted and the dilution rate was then increased until glucose was again no longer limiting. The process was repeated as often as necessary. The composition of the continuous culture medium was tested for optimal promotion of growth in this manner and the critical dilution rate was determined.

This method of nutrient screening seemed preferable in our system to adding pulses to the media as concentrated nutrient solutions. The method produced a slow increase in nutrient concentration in the reactor vessel and the dissolved oxygen concentration could be monitored continuously for any prospect of detrimental effect due to trace metal toxicity. When such an effect was noted the culture could be saved by rapidly restoring the flow from the original reservoir and testing each nutrient separately for toxicity.

VIII. Methods of Extraction of Macromolecules.

A. Protein.

A culture sample of 2 ml was mixed with 8 ml of cold 10% (w/v) TCA and allowed to stand for 10 min. in an ice bath. The mixture was centrifuged for 15 min. at 12,000 x g in a refrigerated centrifuge and the supernatant fluid was discarded. The pellet was thoroughly mixed with 9 ml of cold 10% TCA then incubated at 80°C for 1 hour. The mixture was centrifuged as described previously and the supernatant fluid was discarded. The pellet was then mixed with 8 ml of 0.2 N NaOH and incubated at 100°C for 15 min.

B. Nucleic Acids.

A culture sample of 2.5 ml was mixed with an equal volume of cold 10% TCA, centrifuged, and the supernatant fluid was discarded. The pellet was mixed with 5 ml of cold 0.25 N perchloric acid and was allowed to stand for 30 min. in an ice bath with occasional stirring. The mixture was centrifuged and drained and the pellet was suspended in 4 ml of 0.5N perchloric acid. This mixture was placed into a 70°C water bath and stirred occasionally. After 20 min. the mixture was

centrifuged and the extract was collected. The extraction technique was repeated twice using 3 ml volumes of perchloric acid and the extracts were combined to give a final volume of 10 ml.

C. Free lipid.

Washed cells were homogenized in a chloroform:methanol solution (1:2 v/v) for 3 min. using a Waring Blender at high speed. The suspension was diluted 1/3 with distilled water, homogenized for an additional minute, and filtered through Whatman #12 filter paper into a separatory funnel. Following separation, the chloroform layer was collected.

IX. Analytical methods.

Glucose, nitrogen, and phosphate concentrations were determined in culture supernatant fluids by the methods of Miller (1959), Weatherburn (1967), and Fiske and Subbarow (1925), respectively. The amount of each material utilized was estimated by subtracting the amount present in the spent medium from the assayed amount present in the sterile uninoculated medium.

Protein values were determined for cellular extracts by the method of Lowry, et al. (1951) using

bovine serum albumin as a standard. Nucleic acid extracts were used for the estimation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) by the diphenylamine and orcinol reactions, respectively (Schneider, 1956), using calf thymus DNA and Torula yeast RNA as standards. Free lipid content was determined gravimetricly by collecting chloroform extracts in preweighed aluminum pans and evaporating to dryness. Also, the carbohydrate content was washed cells was determined by the anthrone reaction (Herbert, et al., 1971).

It was observed by spectrophotometric analysis of a dilute copper sulfate solution in place of medium that a minimum of 6 residence times was required to establish a physical steady state in the reactor vessel following any change of concentration in the medium reservoirs. Thus, the culture was allowed a minimum of 8 mean residence times to reestablish a steady state following any disturbance to the system such as sampling or an alteration in medium composition. Carbohydrate and lipid estimations were only performed upon glucose-limited cultures. All assays, except free lipid estimations, were conducted in triplicate and mean values are presented. Standard deviations were not

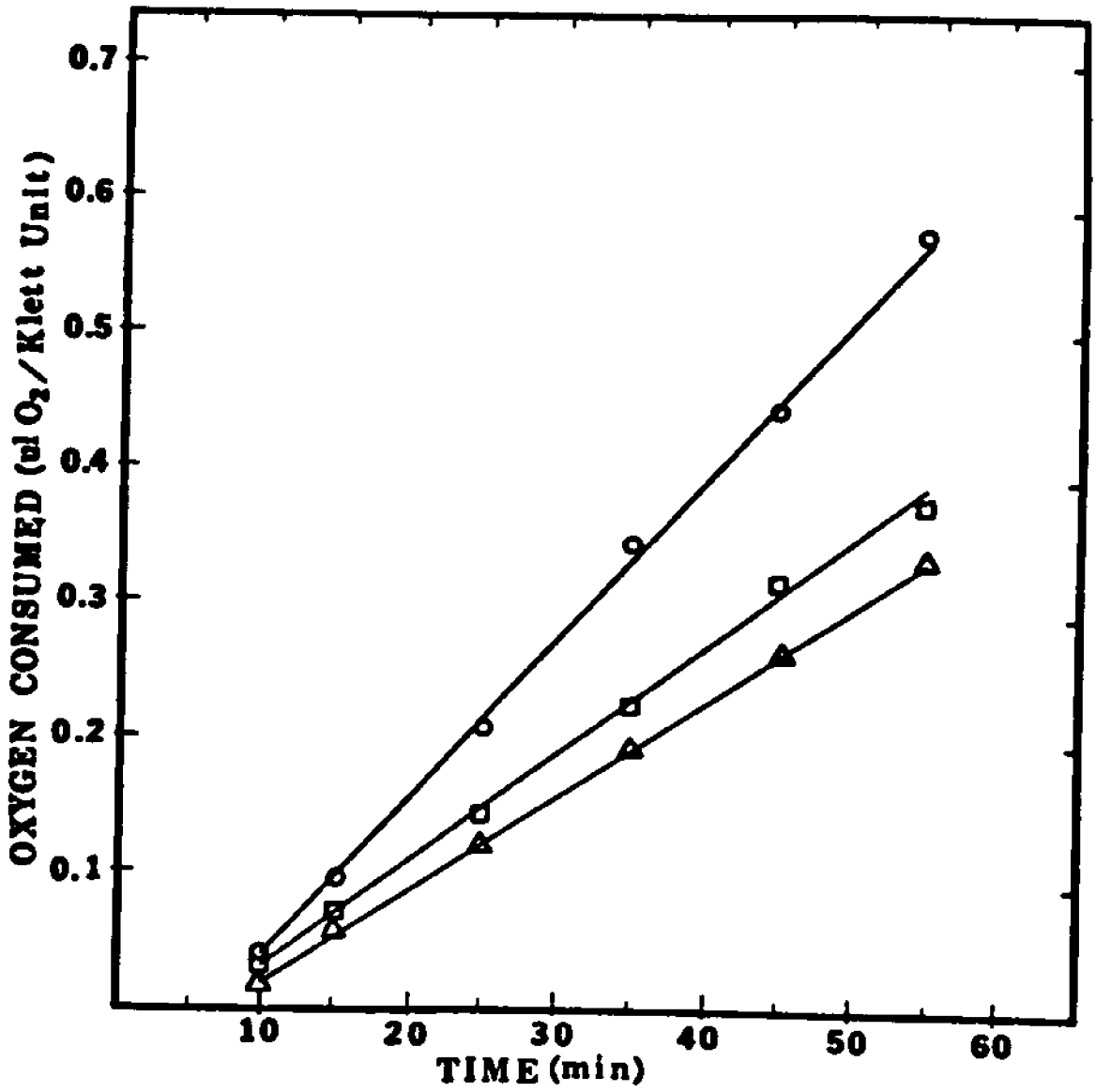
calculated, however, no variation greater than 5 per cent from the reported mean values was noted within samples.

RESULTS

I. Respirometry Studies.

Early attempts towards medium optimization were conducted using Cellulomonas and a variety of techniques under batch growth conditions. Preliminary respirometry studies indicated that this method would not be acceptable for optimization purposes. However, one interesting observation provided useful information concerning the interaction between the cell density and the medium environment. The experiment was conducted to determine the effect of inoculum size upon the rate of oxygen uptake. As expected, oxygen uptake decreased with a decrease in inoculum density. When the results were standardized as shown in Fig 2 it was clear that the rate of oxygen uptake was influenced by the population density and suggested the nutrient concentrations were not in balance with the population density. A similar trend was noted if standardization was made with viable cell count instead of optical density (data not shown). The evidence suggested that a very sensitive relationship existed between Cellulomonas and its culture environment.

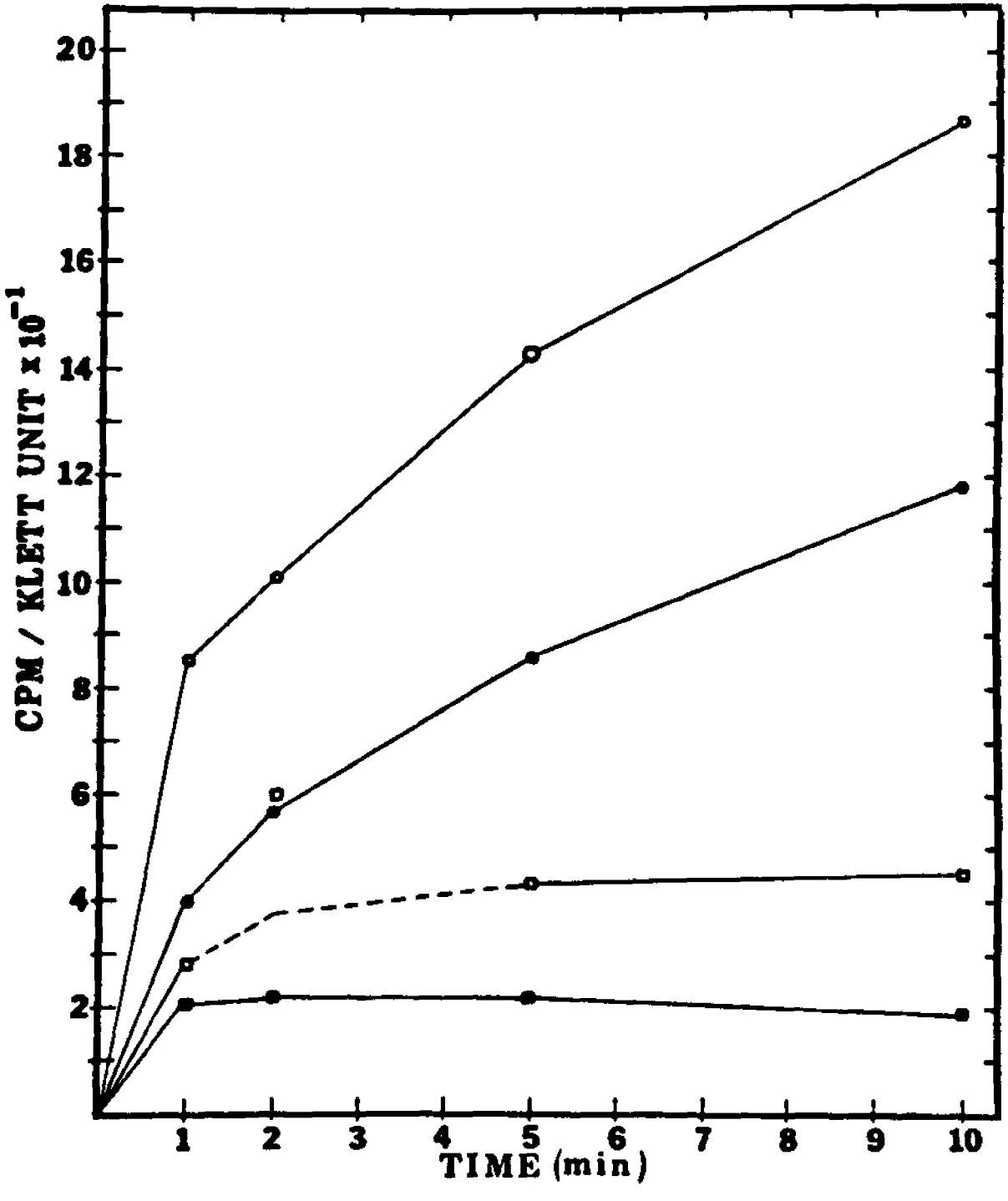
Figure 2. Oxygen uptake of Cellulomonas sp. standardized to Klett unit of culture. Absorbance of 500 Klett unit culture (open circles); 300 Klett unit culture (open squares); 170 Klett unit culture (open triangles).


OXYGEN CONSUMED (ml O2/Klett Unit)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
10
20
30
40
50
60
TIME (min)

II. Ribonucleic acid synthesis.

These studies were also designed as an attempt to develop a model system for use as a means of medium optimization in batch culture. Once a culture has reached expoential phase growth repeated transfer into fresh medium usually results in a condition described as balanced growth. When the population is undergoing balanced growth the growth rate is constant. In addition, macromolecular synthesis, e.g. DNA, RNA, and protein synthesis, is also maintained at a constant rate. This appeared to be a promising means for screening a variety of medium components for stimulation of RNA synthesis during balanced growth. As a control, the rate of RNA synthesis from lag, early log, late log and early stationary phase cells was examined under conditions in which the log phase cells should have demonstrated balanced growth. The standardized results presented in Fig. 3 clearly indicate balanced growth was not accomplished with Cellulomonas under these conditions. After the addition of cells to the fresh medium the rate of RNA synthesis diminished between 1 to 2 min. The reduction may have actually occurred instantaneously and gone undetected since the first sample was withdrawn after 1 min.

Figure 3. Rate of RNA synthesis of Cellulomonas sp. standardized to Klett unit of the culture. Lag phase cells (closed squares); Early log phase cells (open squares); Late log phase cells (closed circles); Early stationary phase cells (open circles).


20
18
16
14
12
10
8
6
4
2
CPM / KLETT UNIT x 10⁻¹
1
2
3
4
5
6
7
8
9
10
TIME (min)

Both lag and early log phase cells completely terminated RNA synthesis within 5 min. and by 10 min. late log and early stationary cells had nearly halted synthesis. During the course of this experiment it was interesting to note that the initial rate of RNA synthesis increased with the culture age, which was unexpected. Since balanced growth was not achieved under these conditions another means of medium optimization was searched for.

III. Batch growth characteristics of Cellulomonas LC-10.

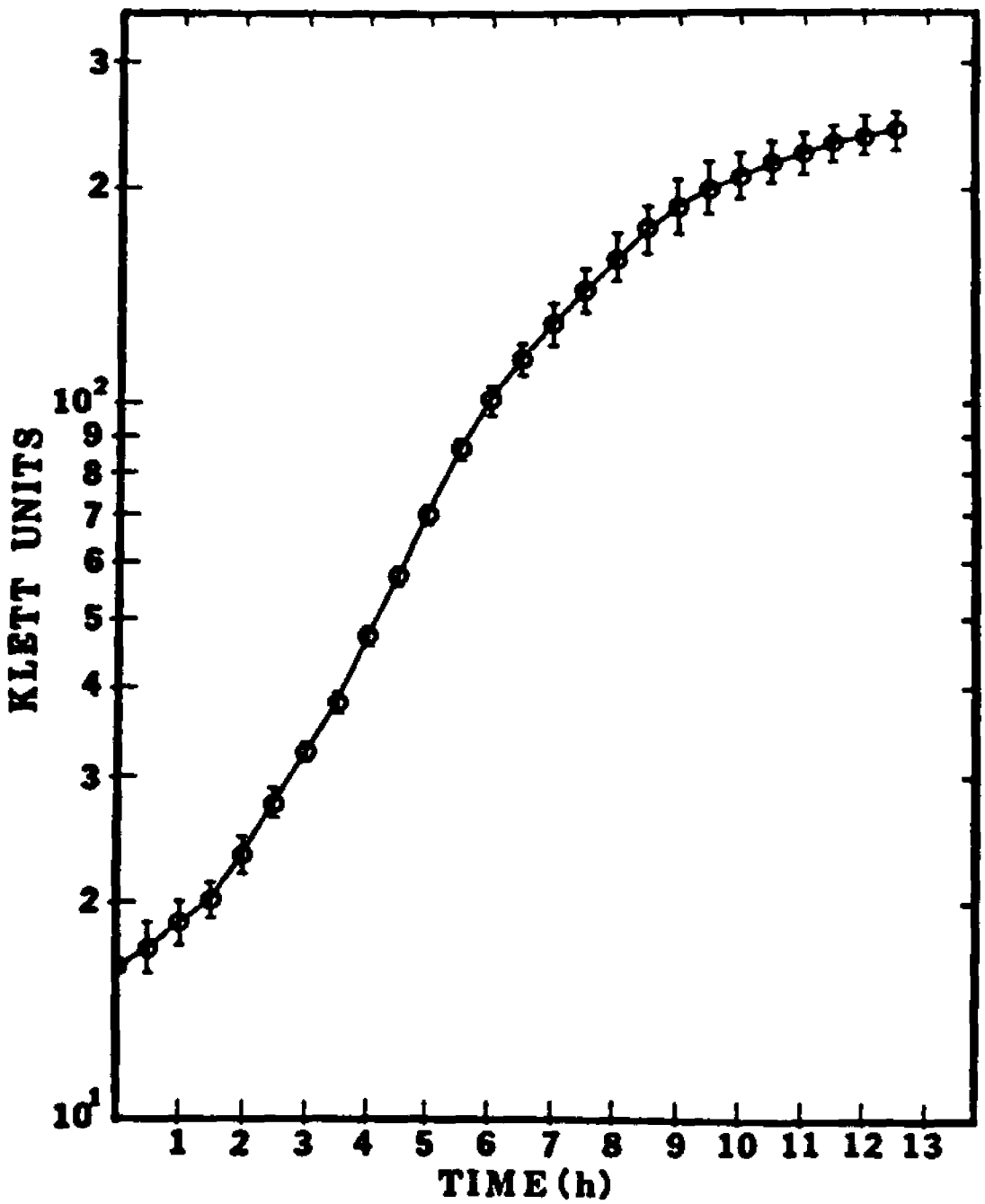
Cellulomonas LC-10 was isolated as a spontaneous mutant derived from the parent strain by Choi (1976). The organism grows on all three defined media described in Table 1, exhibiting a wide range of generation times. The large difference between CH-medium and A-medium may be due solely to the addition of D-pantothenic acid or a concerted effect by a number of alterations. The subtle differences between the A and B media continued to influence the growth rate, but the yield remained the same (data not shown). A series of batch growth experiments were conducted using LC-10 and B-medium. The organism was passed through the medium twice as starter cultures. Mid-log phase cells from the second passage

were used as inocula into 5 side arm flasks (250 ml), each containing 100 ml of sterile medium. Each flask was inoculated at a starting density of 18 Klett units and turbidity measurements were conducted every 30 min. during incubation. The batch growth curve from the averaged data, including the standard deviations for each point, is presented in Fig. 4. Following inoculation the organism passed through a phase of accelerated growth for a period of 4 to 4.5 h. The exponential phase was rather brief, lasting for approximately one generation time, followed by a period of decelerating growth rate. The cellular population had reached stationary phase by 13 h. Microscopic examination of the cells immediately following inoculation revealed an interesting fact. The cells very rapidly clumped together forming large aggregates. Within two hours following inoculation the cells had reestablished the single random grouping characteristic of Cellulomonas and remained so for the remainder of the growth cycle.

IV. Medium optimization by continuous cultivation.

The ingredients comprising B-medium were selected for use in the chemostat since the organism demonstrated its most rapid growth rate in this medium. Prior to

Fig. 4. Batch growth cycle of Cellulomonas LC-10 cultivated in B-medium at 30°C and 200 RPM.


KLETT UNITS
3
2
10²
9
8
7
6
5
4
3
2
10¹
1 2 3 4 5 6 7 8 9 10 11 12 13
TIME (h)

chemostat studies, typical nutritional studies were conducted in batch using various concentrations of trace metals to provide guidelines toward reading medium optimization in continuous culture. The results of these studies are presented in Table 2. Magnesium chloride appeared to be in optimum concentration with respect to the other nutrients present in B-medium. However, the generation time was improved by either increasing or decreasing the concentration of each of the other trace metals. It was interesting to note the variation in generation time observed using the normal B-medium (1.51 - 2.45). These differences between experiments were probably due to differences in the age of the washed cells harvested from starter cultures. The results observed in these experiments were used as a guide for constructing a medium for use in the continuous cultivation of Cellulomonas.

Certain components of B-medium, such as $(NH_4)_2SO_4$, K_2HPO_4, $NaH_2PO_4 \cdot H_2O$, and $CaCl_2 \cdot 2H_2O$, were believed to be present in excess. In an effort to devise a lean medium for continuous cultivation, concentrations of these components were subsequently diminished. Trace metal concentrations were adjusted according to the trends obvious in Table 2. The original continuous culture medium was

Table 2. Effect of varying trace metal concentrations upon the generation time of Cellulomonas LC-10 grown in batch culture.

Ingredient	Generation Time (h)					
	10x[a]	5x	1x	0.5x	0.1x	0.01x
$MgCl_2 \cdot 6H_2O$	1.65	1.60	1.60	2.28	1.85	2.83
$FeSO_4 \cdot 7H_2O$	2.84	2.49	2.45	2.07	1.99	2.77
$ZnSO_4 \cdot 7H_2O$	1.85	1.71	2.16	2.66	2.96	3.59
$CoCl_2 \cdot 6H_2O$	2.32	2.01	1.97	1.87	2.03	1.52
$CuSO_4 \cdot 5H_2O$	2.10	2.07	1.51	1.87	1.72	1.50
$MnCl_2 \cdot 4H_2O$	1.59	1.37	1.75	1.67	1.54	1.53

[a]Concentration of 1 nutrient was varied while all others were maintained at 1x relative to B-Medium.

constructed in Table 3 and supported a dilution rate (D) of 0.1 h^{-1}. When D was increased to 0.175 h^{-1}, glucose was no longer growth limiting. The growth limiting nutrients were identified as nitrogen, phosphate, and zinc. The concentrations of these materials were increased to the values listed in Table 3 and the glucose limited steady state was restored. This process was repeated for D's of 0.240 h^{-1}, 0.398 h^{-1}, and 0.476 h^{-1}. The final presumably optimized medium supported a steady state population until D exceeded 0.500 h^{-1}. However, a final zinc sulfate concentration of 1×10^{-3} g/liter was required to maintain steady state until washout was achieved at a D of 0.571 h^{-1}. The critical dilution rate in continuous culture approximated the average maximum specific growth rate estimated from batch culture. Also, a zinc sulfate concentration of 1.15×10^{-3} g/liter was found to be toxic to the organism at all growth rates. During the optimization of this medium, Klett unit values of cellular suspensions from glucose-limited steady states varied between 750 to 800.

V. Glucose-limited continuous cultivation.

The nutrient concentrations of the presumably optimized medium for the growth of Cellulomonas LC-10 is

Table 3. Apparent optimization of continuous culture medium for Cellulomonas LC-10 by overcoming nutrient limitations at increasing dilution rates.

Ingredients	Concentration (g/liter)				
Glucose	5				
$(NH_4)_2SO_4$	0.75	0.975	1.5		
K_2HPO_4	0.18	0.234		0.36	
$NaH_2PO_4 \cdot H_2O$	0.06	0.078		0.12	
$CaCl_2 \cdot 2H_2O$	0.01				
$MgCl_2 \cdot 6H_2O$	0.1				0.125
$FeSO_4 \cdot 7H_2O$	2.5×10^{-3}				
$ZnSO_4 \cdot 7H_2O$	2×10^{-4}	6×10^{-4}	8×10^{-4}		
$CoCl_2 \cdot 6H_2O$	2×10^{-5}				
$CuSO_4 \cdot 5H_2O$	5×10^{-7}				
$MnCl_2 \cdot 4H_2O$	1×10^{-5}				2×10^{-5}
Thiamine HCl	5×10^{-6}				
D-Pantothenic Acid	5×10^{-6}				
D-Biotin	2×10^{-7}				
Dilution Rate (h^{-1})	0.100	0.175	0.240	0.398	0.476

presented in Table 4. The medium does not contain a zinc sulfate concentration sufficient for attainment of the critical dilution rate, but will support glucose limited growth at a D slightly greater than 0.400 h^{-1}. The growth characteristics of the organism upon the improved medium were examined in continuous culture over a wide range of D's and the results are presented in Table 5. It was interesting to observe that under carbon limitation the Klett unit and dry weight steady state values decreased with increased D values. This resulted in a decrease in the glucose yield coefficient (Y_{GLU}) from 0.69 to 0.49 for D's of 0.100 h^{-1} to 0.400 h^{-1}. Accompanying the reduction in biomass was a relative increase in the phosphate and nitrogen consumption of the population. Graphic presentation of the culture characteristics (Fig. 5) indicated a similar trend was also observed with colony forming units. Each of these parameters best fit a bilinear function with a change in slope occurring at a D equal to 0.285 h^{-1}. Detectable amounts of glucose were not present in the culture at any sample points. In addition, the reduction in biomass was not due to cellular adhesion or selective washout within the reactor vessel. Microscopic examination of the culture samples from each D tested revealed no observed differences in cellular morphology or grouping, and clumping was not noted.

Table 4. Final nutrient concentrations of the presumably optimized medium for the growth of Cellulomonas LC-10 in continuous culture.

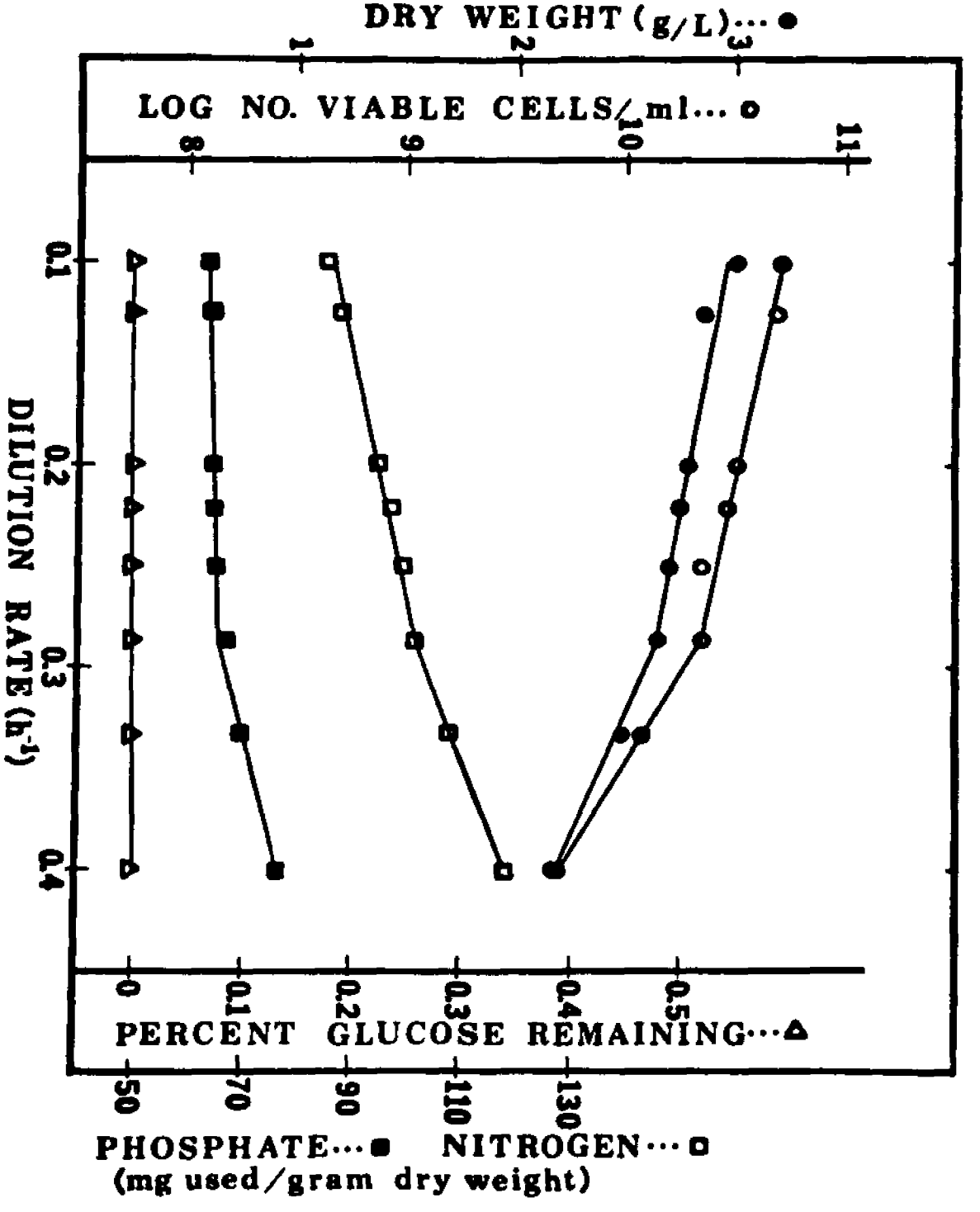
Ingredients	Concentration (g/liter)
Glucose	5
$(NH_4)_2SO_4$	1.5
K_2HPO_4	3.6×10^{-1}
$NaH_2PO_4 \cdot H_2O$	1.2×10^{-1}
$CaCl_2 \cdot 2H_2O$	1×10^{-2}
$MgCl_2 \cdot 6H_2O$	1.25×10^{-1}
$FeSO_4 \cdot 7H_2O$	2.5×10^{-3}
$ZnSO_4 \cdot 7H_2O$	8×10^{-4}
$CoCl_2 \cdot 6H_2O$	2×10^{-5}
$CuSO_4 \cdot 5H_2O$	5×10^{-7}
$MnCl_2 \cdot 4H_2O$	2×10^{-5}
Thiamine HCl	5×10^{-6}
D-Pantothenic Acid	5×10^{-6}
D-Biotin	2×10^{-7}

Table 5. Yield and macronutrient consumption by Cellulomonas LC-10 grown continuously on the apparently optimized medium at a variety of dilution rates.

Dilution Rate (h^{-1})	0.100	0.125	0.200	0.222	0.250	0.285	0.333	0.400
Klett Units	942	895	830	805	780	755	760	710
Dry Weight (g/liter)	3.21	3.19	3.02	2.97	2.85	2.85	2.58	2.45
Y_{GLU}[a]	0.64	0.64	0.60	0.59	0.57	0.57	0.52	0.49
mg Phosphate used/ g Dry Weight	63.90	64.28	64.40	64.84	65.62	67.27	69.39	75.80
mg Nitrogen used/ g Dry Weight	85.48	87.96	94.47	97.28	99.60	101.58	108.08	118.26

[a] Y_{GLU} is defined as the yield constant or weight of bacteria formed per unit weight of glucose used.

Figure 5. Population growth characteristics of Cellulomonas LC-10 grown in glucose-limited continuous culture.

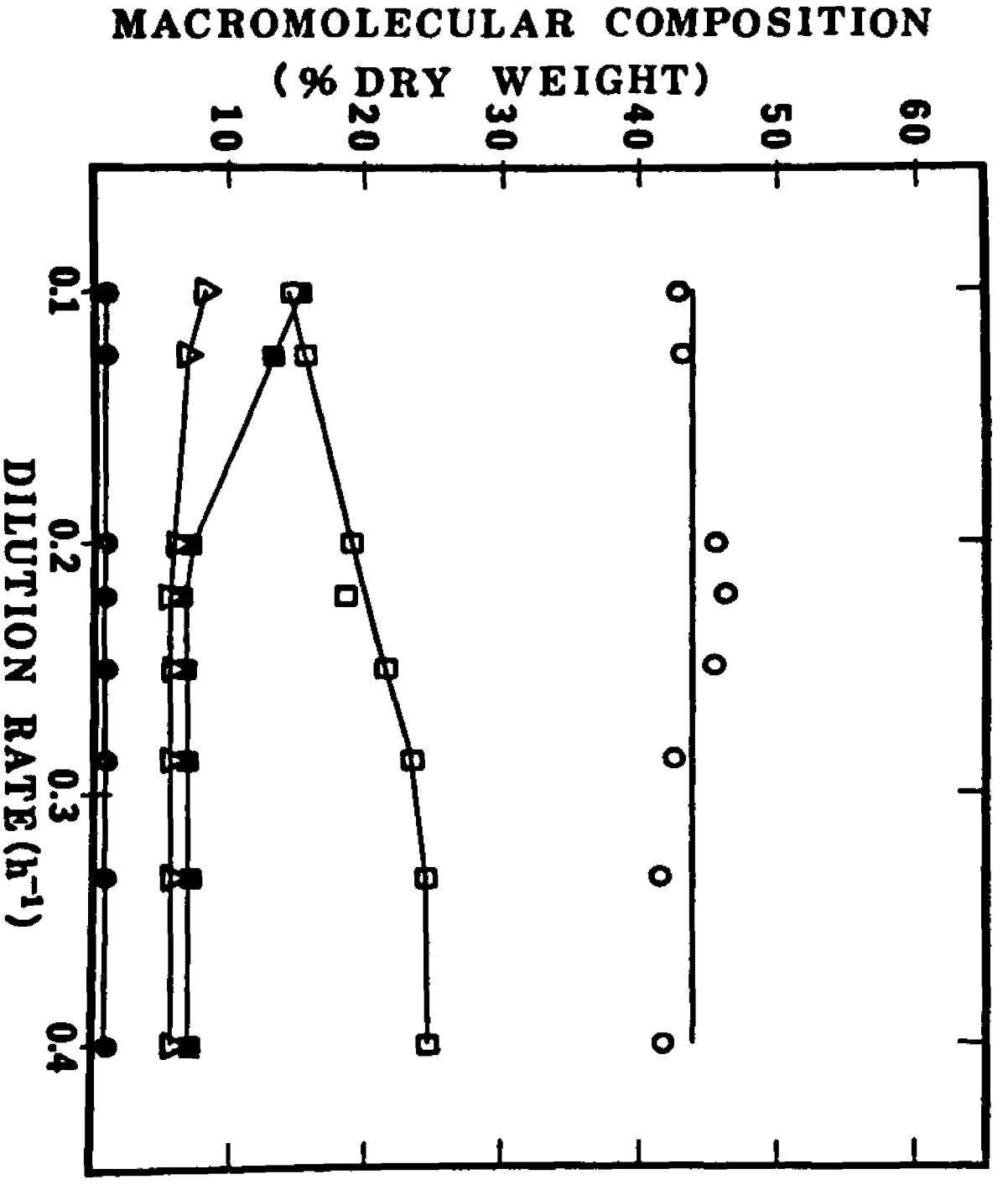

DRY WEIGHT (g/L)... ●
1
2
3
LOG NO. VIABLE CELLS/ml... ○
8
9
10
11
DILUTION RATE (h⁻¹)
0.1
0.2
0.3
0.4
0
0.1
0.2
0.3
0.4
0.5
PERCENT GLUCOSE REMAINING... △
50
70
90
110
130
PHOSPHATE... ■ NITROGEN... □
(mg used/gram dry weight)

The relative macromolecular composition of the cells in the population was determined for these growth conditions and is presented in Fig. 6. The protein and free lipid content was found to remain constant at all D values and accounted for approximately 45 and 1 per cent of the biomass, respectively. However, the nucleic acid and carbohydrate contents were found to undergo changes which were dependent upon D. The RNA content increased from 14 per cent (0.10 h^{-1}) to 24 per cent (0.333; 0.40 h^{-1}). The increase in RNA was linear until D equaled 0.285 h^{-1}. Carbohydrate and DNA diminished from 15 to 7 per cent and 8 to 5 per cent, respectively, and remained constant for D values of 0.222 through 0.40 h^{-1}. The estimations accounted for approximately 80 to 85 per cent of the biomass at all D values.

VI. Zinc-Limited Continuous Cultivation.

It has been reported previously that zinc concentration plays a significant role in the regulation of growth (White and Johnson, 1971; Wilson and Reisenauer, 1970; Wold and Suzuki, 1976) and RNA synthesis (Cocucci and Rossi, 1972; Falchuk, et al., 1978). Therefore, the effect of zinc limitation upon the growth rate and macromolecular composition of Cellulomonas LC-10 grown

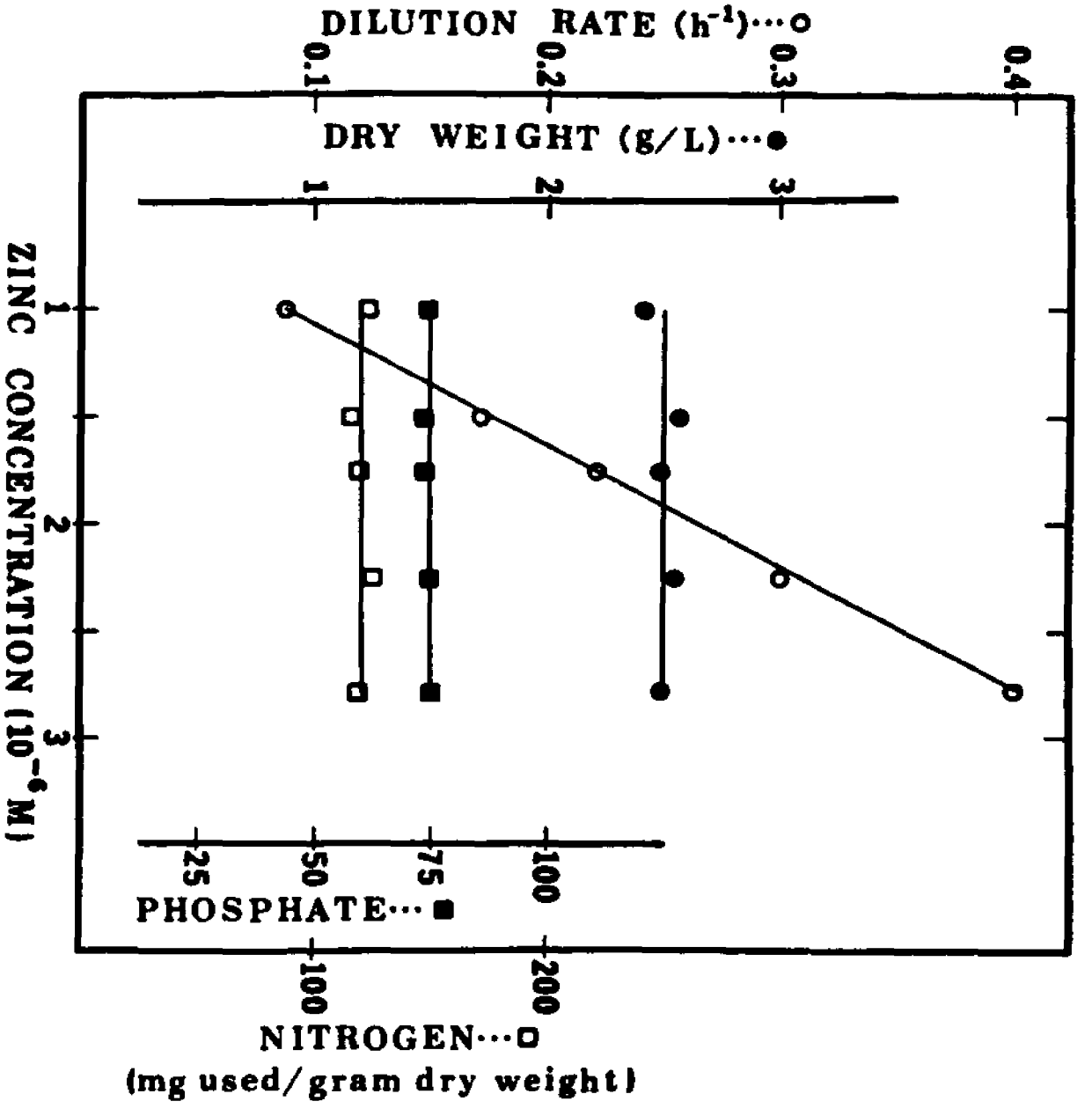
Figure 6. Relative macromolecular composition of Cellulomonas LC-10 grown in glucose-limited continuous culture. Protein (open circles); RNA (open squares; Carbohydrate (closed squares); DNA (open triangles); Free lipid (closed circles).


MACROMOLECULAR COMPOSITION
(% DRY WEIGHT)
10
20
30
40
50
60
0.1
0.2
0.3
0.4
DILUTION RATE (h⁻¹)

in continuous culture was examined. This was accomplished by reducing the feed concentration of zinc in the medium. When the zinc concentration was reduced, the biomass decreased significantly and glucose was detected in the medium. However, if the D value was reduced, biomass and glucose utilization increased. The D value was adjusted to a rate where only a trace amount (approximately 10 ug/ml) of glucose was detected in the effluent. Thus, nearly all the glucose was utilized and the D value was termed the maximum specific growth rate achievable for that zinc concentration. It should be pointed out that it is not clear that these results are due solely to zinc limitation. Since zinc is a cofactor for a wide number of enzymes the reduced concentration may be limiting the population at either the rate of glucose uptake or metabolism. This would therefore be imposing a dual zinc and glucose limitation upon the population.

The culture growth characteristics of Cellulomonas were entirely different when cultivated under these conditions (Fig. 7). The maximum specific growth rate achievable is directly proportional to the zinc concentration in the medium. The biomass, phosphate and nitrogen utilization remained constant over the

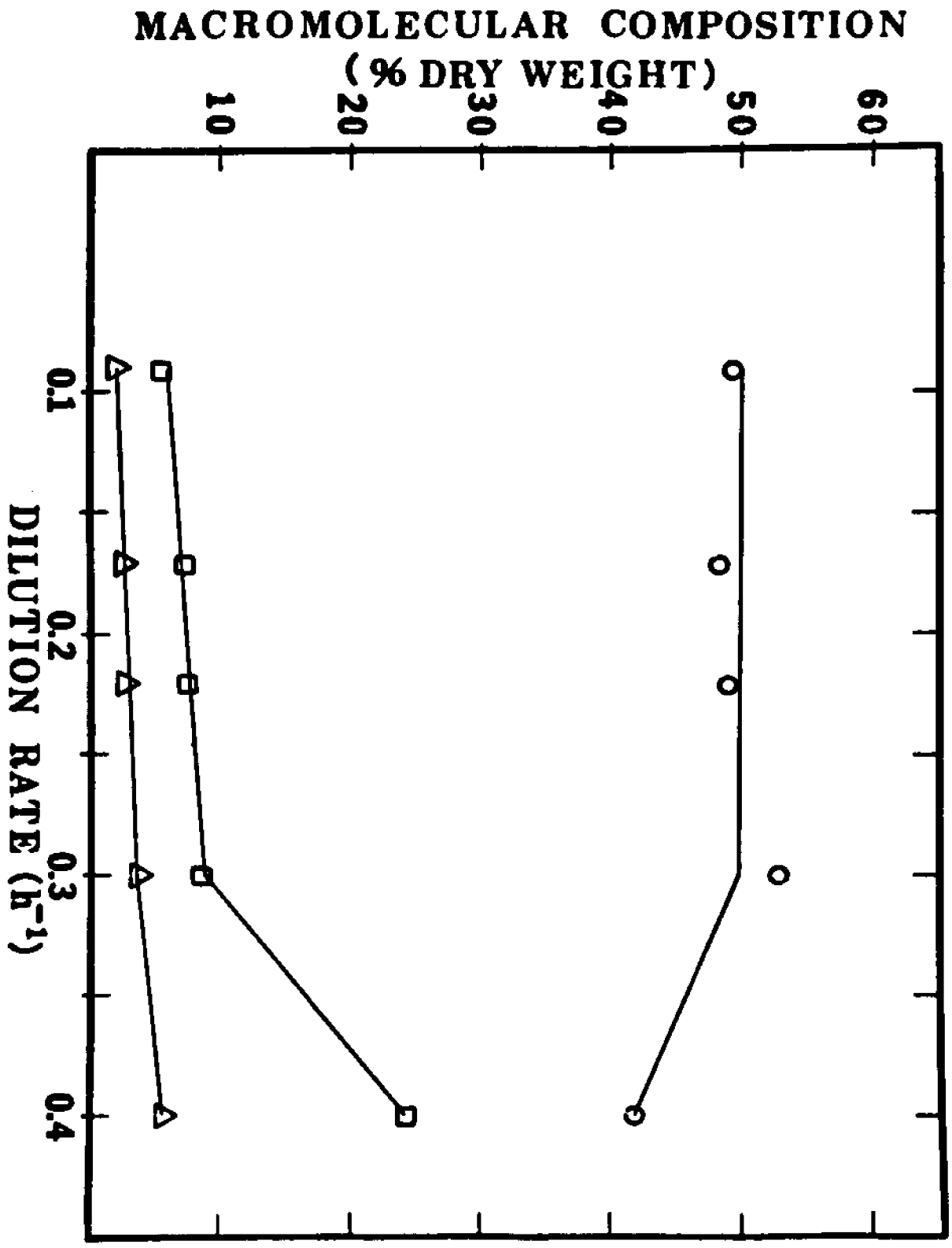
Figure 7. Variation of dilution rate and population growth characteristics dependent upon zinc concentration for obtaining steady state yields from a specified amount of glucose in continuous culture. At a zinc concentration of 2.87 uM and D value of 0.40 h^{-1} the organism is growing under glucose limitation on the complete medium.


DILUTION RATE (h^{-1})···○
0.1
0.2
0.3
0.4
DRY WEIGHT (g/L)···●
1
2
3
ZINC CONCENTRATION (10^{-6} M)
1
2
3
25
50
75
100
PHOSPHATE···■
100
200
NITROGEN···□
(mg used/gram dry weight)

range of zinc concentrations examined. These values were equal to values estimated from glucose limited cultures using the optimized medium at a D equal to 0.40 h^{-1}.

Since the population growth characteristics varied between glucose and zinc-limited cultures and the maximum specific growth rate was proportional to zinc concentration, it proved interesting to examine the macromolecular composition of zinc-limited cells. The cells were cultured continuously at the maximum specific growth rate achievable for various zinc feed concentrations and relative protein, RNA, and DNA estimations are presented in Fig. 8. The values plotted for a D value of 0.40 h^{-1} were taken from the optimized medium under conditions of glucose limitation. The protein content of zinc limited cells was slightly elevated, but remained constant over a range of 1 to 2.24 uM zinc. The nucleic acid content of the population was significantly reduced under the zinc limitation and increased linearly with increased zinc concentration. It was also interesting to note the difference in the profile of relative DNA as a function of D in glucose-limited (Fig. 6) and zinc-limited (Fig. 8) cultures. Increased relative amounts of DNA were not observed for D values less than 0.20 h^{-1} in zinc-limited cultures.

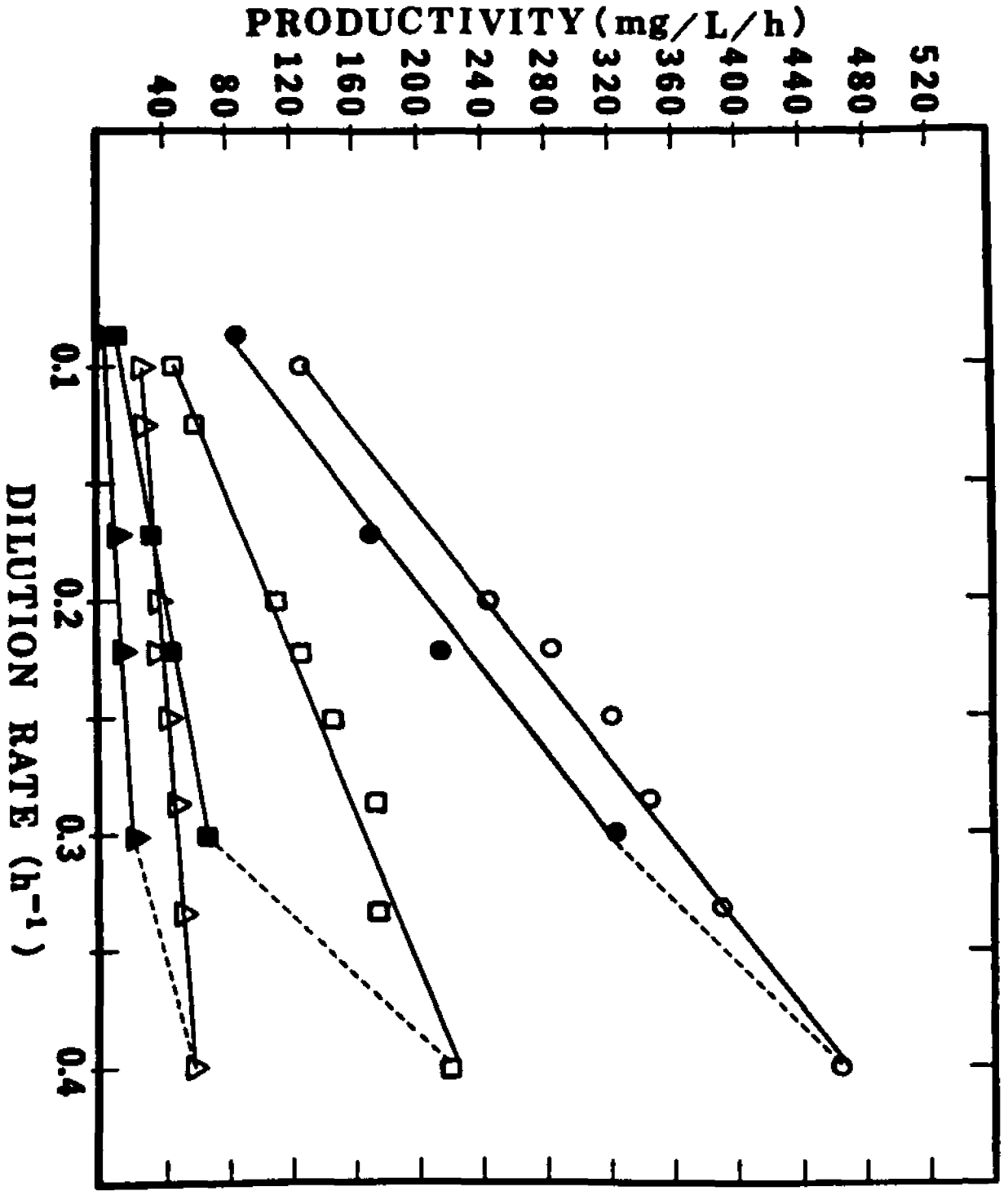
Figure 8. Relative protein (circles), RNA (squares), and DNA (triangles) composition of Cellulomonas LC-10 grown in zinc-limited continuous culture. Zinc concentrations of 1, 1.5, 1.75, and 2.25 uM enabled maximum D values of 0.086, 0.172, 0.222, and 0.303 h^{-1}, respectively. At a zinc concentration of 2.78 uM and a D value of 0.400 h^{-1} the culture is growing under glucose limitation on the complete medium.


MACROMOLECULAR COMPOSITION
(% DRY WEIGHT)
10
20
30
40
50
60
DILUTION RATE (h⁻¹)
0.1
0.2
0.3
0.4

VII. Comparison of glucose and zinc-limited cultures.

The culture growth characteristics and relative amounts of DNA and RNA were clearly different between glucose and zinc-limited cultures. However, it is more meaningful from a quantitative aspect to compare the data on the basis of productivity (absolute amount produced/L/unit time). The protein, RNA, and DNA productivity values from both culture conditions were plotted as a function of D (Fig. 9). The protein productivity of zinc-limited cultures was slightly depressed compared to values obtained from glucose-limited cultures. However, these values increased in parallel with values from glucose-limited cultures as a function of D. Conditions of zinc limitation resulted in a significant reduction in nucleic acid productivity of the population. The results of this work would appear more dramatic if the protein to RNA ratios were compared between glucose and zinc-limited cultures. Over the D range from 0.1 h^{-1} to 0.4 h^{-1} this ratio decreased linearly from 2.88 to 1.89 for glucose limiting conditions. This ratio is significantly higher for zinc limitation. For example, at the lowest D value for zinc-limited growth (0.086 h^{-1}) this ratio was 7.68. For the moderate D value of 0.25

Figure 9. Protein (circles), RNA (squares), and DNA (triangles) productivity values from glucose-limited (open symbols) and zinc-limited (closed symbols) cultures plotted as a function of dilution rate.


PRODUCTIVITY (mg/L/h)
40
80
120
160
200
240
280
320
360
400
440
480
520
DILUTION RATE (h⁻¹)
0.1
0.2
0.3
0.4

h^{-1}, the protein to RNA ratio was 5.08 compared to a ratio of 2.20 for glucose-limited cells.

VIII. Gradient-feed technique.

High cell yields at a rapid rate are required to make an industrial process for the production of single-cell proteins economical enough to compete with the cost of presently available proteins. The previous studies on the nutrition and physiology of Cellulomonas LC-10 were carried out in order to determine the optimum conditions for increasing the productivity. The results of the continuous culture studies produced a presumably optimized medium which is in careful balance with the cell population. Therefore, it is a reasonable assumption that the yield of a growing population can be increased by increasing the concentration of all nutrients at a rate near the growth rate of the population. This assumption was tested in the following manner.

Four and one half liters of the medium were inoculated with sufficient organisms so that a growth of 500-700 Klett units (approximately 1.5-2.0 g/liter of dry weight of cells) was obtained during 10-12 hr. The organisms were grown in a New Brunsiwck 7 liter fermentor equipped with a steam-sterilizable pH electrode. The pH of

the culture was maintained at 6.8 by the addition of 2N NaOH using a Radiometer TTTI autotitrator. The temperature of the culture was kept constant at 35°C. During the course of experiments for obtaining high cell densities, the cultures were aerated with 2 vol./min. of air. Air was dispersed by mechanical agitation by maintaining the impellor at 500 rpm. The fermentor was provided with a foam probe and foaming was controlled automatically during fermentation by the addition of polyglycol P-2000 antifoam. Concentrated nutrients were pumped into the fermentor in such a manner that the concentration of nutrients increased linearly with time. A simple gradient mixer usually used in column chromatographic separations in the laboratory was used to generate a linear gradient of the concentration of nutrients (Fig. 10). Although only one gradient is shown schematically, in actual practice, two similar gradients are used. In a typical experiment, the final concentration of nutrients coming into the fermentor was sufficient to give 10 times the initial concentration in the fermentor. In the experiment described here a total of 250 g of glucose and proportionate quantities of all the nutrients were fed as a gradient. In order to avoid precipitation of salts in a concentrated solution two separate gradients were

Figure 10. Schematic outline of introducing increasing concentration of nutrients as a linear gradient into the fermenter vessel. 1. Vessel containing high concentration of nutrients. 2. Vessel containing low concentration of nutrients. 3. Peristaltic pump. 4. Fermenter vessel. 5. Air inlet. 6. Air outlet. 7. Impeller. 8. Nutrient inlet.

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used (Bauer and Shiloach, 1974). Nitrogen and phosphorus contents were mixed together in one gradient and the trace minerals in the other. Glucose was added into both the gradients. The vessel containing high concentrations contained 200 g of glucose and corresponding amounts of other nutrients while the vessel containing low concentrations had 50 g of glucose with proportionate amounts of the others. The total time of pumping was 10 hr. The total volume of the additional nutrients was kept between 500-600 ml. The increase in volume of the culture was approximately 10% of the original volume present in the fermentor. Growth was monitored by taking samples at intervals and measuring the dry weight and the turbidity after suitable dilution.

Table 6 presents the results of one of several experiments on the growth of Cellulomonas LC-10 by the technique described. A final yield of 29.4 g dry cell mass/liter was obtained which was equivalent to approximately 150 g wet cell wt./liter.

Table 6. Growth of Cellulomonas LC-10 by the Gradient-Feed Technique in a 7 L. Fermentor[a]

Time (h.)	Absorbance (Klett units)	Dry wt (g/liter)
t_0	700	2.1
t_2	1850	5.5
t_3	2600	7.8
t_5	4200	12.8
t_{10}	9800	29.4

[a]A total increase of 14-fold with an average doubling time of 2.63 was obtained.

DISCUSSION

Environmental factors influence all aspects of physiology and regulation. Chemically defined media have been employed to study the effect of different nutrients on reactions related to the growth cycle of many organisms. The importance of proper nutrient balance to the population density is recognized and has added to the ease of scale up operations in industrial fermentations. Members of the genus Cellulomonas exhibit cellulolytic activity and prove to be of interest as a potential source of SCP from cellulose fermentation. However, an understanding of the nutrition and physiology of growth related processes is required before a cellulose fermentation unit can be designed.

Continuous cultivation has been used successfully to study growth related phenomena. This technique can be accomplished easily when all nutrients are soluble in the medium. However, at the bench level, the use of an insoluble substrate, such as cellulose, results in a number of mechanical difficulites. Continuous pumping often results in clogging of the nutrient lines, an unequal distribution of substrate particles, and an

imbalance of free cells to cells bound to the cellulose fibers. We have found from our experience that standardizing a medium against glucose requires little modification when changing to a cellulosic substrate. Therefore, the use of glucose as a carbon and energy source can provide information concerning the change in growth response of the population in relation to growth rate. In addition, an estimate of the growth potential of this organism may be achieved which could influence the desirability of choice for use in such a fermentation.

Previously, all attempts to cultivate Cellulomonas LC-10 continuously using conventional batch media proved unsatisfactory. For this reason, the most promising batch culture medium (B-medium) was selected for optimization studies leading to the development of a lean apparently optimized defined medium.

Recently, statistical modeling techniques have been proposed for multi-dimensional rapid optimization of microbiological media (Maddox and Richert, 1977; Voltruba, et al., 1975). These techniques have proved successful, but the aid of computer analysis was desired and caution was expressed in dealing with three-factor or higher interactions commonly encountered in biological systems (Maddox and Richert, 1977). Mateles and Battat (1974) devised a technique whereby continuous culture

was used as a tool for optimization of a medium for the growth of Pseudomonas C on methanol. A residence time of approximately 3.1 h was selected to grow the organism. At this residence time methanol was not the growth limiting nutrient. Once the culture achieved steady state, concentrated solutions of individual components were injected into the growth vessel. Shortly after the disturbance to the culture, samples were assayed spectrophotometrically for an increase in culture density during the transient state. In this manner a variety of nutrient limitations were identified and later overcome to design a fully optimized medium for the growth of the organism at that dilution rate. Other workers have used this technique to confirm the presence of adequate nutrient concentrations of a basal medium for a desired biomass (Ornelas-Vale, et al., 1977).

The information collected from batch culture suggested Cellulomonas LC-10 was extremely sensitive to the nutritional conditions of the medium. Obviously, changes in the concentrations of trace metals in batch culture affected the growth characteristics of Cellulomonas LC-10, but those methods lacked the sensitivity observed by using continuous culture for optimization. The optimization method described by Mateles and Battat (1974) would have been desirable, had it not been

for the narrow range between the optimum concentration and toxic concentration of zinc. The method used for Cellulomonas LC-10 allowed the population to be saved as soon as toxicity was detected by restoring the original medium reservoirs.

The results obtained with Cellulomonas LC-10 were used as a guide to varying the composition of a Bacillus cereus medium (Boudreaux, 1978). It appeared significant that during the course of optimization of the B. cereus medium the concentrations of $MgCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 7H_2O$ had to be increased with an increase in population growth rate also. Other workers have reported similar findings, but the manner of involvement is complex, and to date is poorly understood (Mueller, 1938; Winder and O'Hara, 1962, Tempest, et al., 1967; Wilson and Reisenaur, 1970; White and Johnson, 1971). Hence, it may be conjectured that the involvement may be due to some common property shared by both microorganisms, such as cofactors involved in glucose metablism, DNA, RNA or protein synthesis.

The population growth response in a chemostat is usually described on the basis of Monod kinetics (Monod, 1949). The validity of the Monod model is based upon a number of assumptions, e.g., the yield coefficient is

constant and independent of growth rate, the population is homologous, and the physiological state of the population remains constant. When Cellulomonas is grown under conditions of carbon and energy limitation the yield coefficient clearly varies. This trend of biomass variation is not believed to be due to the design of the continuous culture apparatus since Boudreaux (1978) did not observe similar findings using B. cereus.

The cause for a change in the yield coefficient is unclear at this time, but may be due to a number of factors. Kurowski and Pirt (1975) reported similar findings from studies of the growth of Agrobacterium tumefaciens in phosphate and manganese-limited cultures. The result was explained as an increased cellular requirement for mineral nutrients at increased growth rates. Our results for Cellulomonas LC-10, however, were only observed for growth on the presumably optimized medium and probably reflects the efficiency of carbon utilization.

Alterations in the macromolecular composition as a function of the specific growth rate are dramatic under conditions of glucose limitation. The decrease in carbohydrate content at increasing D values suggests utilization of reserve carbon to meet an increased energy

demand. This stands to reason since the RNA content of the population increases significantly as a function of D and macromolecular synthesis is an energy requiring process. The change in DNA content may reflect the presence of multiple copies of the genome present in cells at reduced growth rates. This is a phenomenon associated with resting cells in batch culture (Herbert, 1961).

In addition, it has been reported that at specific growth rates less than 0.2 h^{-1} a proportion of viable cells to total cells exists for Escherichia coli grown under carbon limitation in a chemostat. As the specific growth rate is reduced the ratio also decreases. At rates exceeding 0.2 h^{-1} to a maximum of 0.7 h^{-1} the ratio of viable to total cells was 1 (Sawada, et al., 1977). These data indicate that at slow growth rates a homologous population does not exist within the chemostat. A steady state does exist between the ratio of dividing to non-dividing cells. Thus, cells would be dividing at rates greater than the dilution rate under conditions of carbon limitation and a proportion of these cells become non-dividing. This may account for the observation of Malek (1958) that physiological states associated with log phase and stationary phase cells could be observed in continuous culture. This occurrence may account for

the observed decrease in yield and constant relative amount of protein in the population. The protein content of dividing cells may increase, but fewer non-dividing cells would be present at increasing D values.

The population growth characteristics observed under conditions of zinc limitation are equally interesting. The maximum specific growth rate achievable was dependent upon zinc concentration. A reduction in steady state biomass and carbon utilization values could be achieved by increasing the D value in excess of the maximum specific growth rate achievable for each specific zinc concentration (data not shown). It has been shown in synchronously dividing cultures of E. coli that the cellular zinc concentration increases 10 to 15 minutes following cell division (Kung, et al., 1976). Thus, a very sensitive and specific relationship exists between the number of dividing cells and zinc concentration.

The results reported in this paper concerning zinc limitation confirm previous observations on the reduction of RNA content in dividing cells (Wacker, 1962; Cocucci and Rossi, 1972). Wacker (1962) also reported a decrease in protein synthesis, but the amino acid, DNA, and polyphosphate content of cells increased. A decrease in protein synthesis was not observed by

Cocucci and Rossi (1972), however, lipid synthesis was stimulated. Whereas, these results demonstrate an increase in the relative protein content, protein productivity was slightly depressed compared to glucose-limited growth, presumably due to the incomplete utilization of glucose. Zinc deficiency in Euglena has been found to block cell division and it has been suggested that zinc is required for the initiation of DNA synthesis (Falchuk, et al., 1975). In addition, zinc has been found tightly bound to procaryotic and eucaryotic DNA polymerases (Slater, et al., 1971), reverse transcriptases (Auld, et al., 1974; Auld, et al., 1975), RNA polymerase I (Scrutton, et al., 1971; Auld, et al., 1976; Falchuk, et al., 1977), II (Falchuk, et al., 1977), and III (Wandzilak and Benson, 1977), and protein elongation factor I (Kotsiopoulos and Mohr, 1975). Removal of zinc from these metalloenzymes resulted in the loss of catalytic activity and function. Thus, zinc has been determined to be an essential micronutrient involved in macromolecular synthesis. However, it must be kept in mind that the involvement of zinc may not be universal. Bacillus cereus RNA content is not only altered by variation in zinc concentration, but also manganese concentration (Boudreaux, 1978).

The concept of the gradient-feed technique was developed from the experiences of other investigators (Bauer and Shiloach, 1974; Shiloach and Bauer, 1975) and several observations during the studies on continuous culture. It is well known that if all the carbon source was included in the culture medium from the start, a rapid assimilative growth occurs followed by a decreased growth rate, the organism switching on to a dissimilative pathway. Thus it became a common practice, to introduce into the medium all the media constituents excepting the principal carbon source from the beginning and feed the carbon source over a period to the growing culture at such a rate that it was assimilated. One factor which is generally overlooked in such experimentation, is the effect of trace elements on the growth rate of the organism. Results from medium optimization studies suggest that not only a certain concentration of trace elements is necessary to maintain an optimum growth rate but also increased concentration of some of the trace elements leads to a decline in the rate of growth and eventually to the cessation of growth itself. Ideally increasing the concentrations of nutrients exponentially with the growth of the organisms; thus keeping the amount of nutrients per organism constant will enable one to

utilize the maximum potentiality of the organism. However, it has been shown that even with a linear gradient it is possible to grow high cell densities with limited instrumentation in bench-scale fermentors. This gradient-feed method may be adapted to grow several other organisms besides Cellulomonas aerobically to obtain high cell densities in relatively short periods of time.

In conclusion, these results confirm the use of continuous cultivation as a tool for apparent medium optimization. The technique is rapid, sensitive and the initial screening of nutrients may not require the establishment of steady state conditions. However, it is clear that no medium can be presumed to be fully optimized until a steady state population can be supported near the maximum growth rate of the population. Based upon this series of experiments it is evident that the optimization of trace metal concentrations is imperative when designing a lean defined medium. The data from these studies indicate that specific nutrient packages can be constructed to achieve maximum specific growth rates.

The use of zinc-limited continuous cultivation of Cellulomonas appears promising towards the production of SCP with reduced nucleic acid content. Even though protein productivity is depressed, reasonable yields can

be obtained with increased dilution rate. This is promising since biomass production can be increased easily due to the nature of the medium. The defined medium used in this study has been stringently optimized for the complete utilization of 0.5 percent glucose. It has been shown previously that the culture density of Cellulomonas LC-10 can be increased rapidly to a maximum of 30 g/l dry weight by increasing the concentration of all nutrients at a rate near the maximum growth rate of the organism. Thus, from these results it appears feasible that a fermentation process for the production of SCP could be developed using a gradient-feed technique to establish a high cell density culture followed by continuous cultivation under zinc-limitation to produce a desirable product. However, before this can be assured the organism must demonstrate the same potential on cellulosic substrates.

To date, the function of zinc in the macromolecular synthetic processes of Cellulomonas is poorly understood, but continued investigation of metal interactions using continuous culture as an experimental tool may prove beneficial towards the understanding of micronutrient involvement in growth related processes and the biochemical consequences of zinc deficiency.

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Richard Joseph Summers was born May 21, 1944, in Cincinnati, Ohio. He graduated from Woodward High School in Cincinnati in June 1962. He began the University of Cincinnati, University College, in the fall of 1962 and transferred to the Evening College in the summer of 1963. From 1963 to 1965 he continued in Evening College while working as a laboratory technician for Procter and Gamble. He served as a Preventive Medicine Specialist in the United States Air Force from 1965 to 1969. After receiving a Honorable Discharge he received a Bachelor of Science and Master of Science in Biology in 1972 and 1974, respectively, from the University of Dayton, Dayton, Ohio. In August, 1974, he entered the graduate school of Louisiana State University and he is a candidate for the degree of Doctor of Philosophy in the Department of Microbiology.

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EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Macromolecular Synthesis of *Cellulomonas* Grown in Continuous Culture

Approved:

V. R. Srinivasan
Major Professor and Chairman
V. R. Srinivasan

James E. Graybeal
Dean of the Graduate School

EXAMINING COMMITTEE:

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Date of Examination:

July 16, 1979